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Role of the Accessory Gene Regulator (*agr*) in Pathogenesis of Staphylococcal Osteomyelitis

ALLISON F. GILLASPY,[1] SANDRA G. HICKMON,[2] ROBERT A. SKINNER,[2] J. ROBY THOMAS,[3] CARL L. NELSON,[2] AND MARK S. SMELTZER[1]*

Department of Microbiology and Immunology,[1] Department of Orthopaedic Surgery,[2] and Department of Pathology,[3] University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205-7199

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To examine the role of the accessory gene regulator (*agr*) in staphylococcal osteomyelitis, we compared a *Staphylococcus aureus* osteomyelitis isolate (UAMS-1) with a derivative of the same strain (UAMS-4) carrying an inactivated *agr* locus. Virulence was assessed with a rabbit model of acute, exogenous osteomyelitis. Bacteria were delivered by microinjection into the midradial region of the forelimb. After 4 weeks, UAMS-1 was identified in the bone of 12 of 13 rabbits infected with $\geq 2 \times 10^6$ CFU and 5 of 6 infected with $\leq 2 \times 10^5$ CFU. In contrast, UAMS-4 was found in 6 of 13 infected with the higher dose and 1 of 6 infected with the lower dose. Additionally, on the basis of a five-point scale assessing radiographic evidence of disease, rabbits infected with UAMS-1 had average scores of 2.64 ± 0.30 (high dose) and 1.43 ± 0.39 (low dose) while rabbits infected with UAMS-4 had average scores of 0.95 ± 0.23 (high dose) and 0.63 ± 0.20 (low dose). Uninfected controls had an average score of 0.53 ± 0.08. The results obtained with UAMS-1 were significantly different from those obtained with UAMS-4 at both doses ($P \leq 0.047$). The results obtained with UAMS-4 were not significantly different from those obtained with the controls at either dose of UAMS-4 ($P \geq 0.150$). On the basis of a similar five-point scale assessing histopathological evidence of disease, rabbits infected with UAMS-1 had average scores of 2.31 ± 0.22 (high dose) and 1.96 ± 0.36 (low dose) while rabbits infected with UAMS-4 had average scores of 1.58 ± 0.29 (high dose) and 0.83 ± 0.32 (low dose). Controls had an average score of 0.33 ± 0.05. The results obtained with UAMS-1 were significantly different from those obtained with UAMS-4 at both doses ($P \leq 0.040$). However, the results obtained with UAMS-4 were significantly different from the controls only at the high dose of UAMS-4 ($P = 0.025$). We conclude that mutation of *agr* reduces the incidence and severity of disease but does not eliminate the ability to colonize bone and cause histopathological evidence of osteomyelitis.

Despite intensive research efforts and continued advances in the treatment of infectious disease, *Staphylococcus aureus* remains a prominent bacterial pathogen (24, 32). While most *S. aureus* infections are confined to the skin and underlying tissues, the elaboration of extracellular toxins from localized foci of infection can lead to serious and even life-threatening systemic disease (e.g., toxic shock syndrome). Moreover, in the case of highly invasive strains and/or an immunocompromised host, *S. aureus* may overcome the local phagocytic systems and invade the bloodstream (32). Although the ensuing bacteremia can lead to the colonization of essentially any tissue, *S. aureus* exhibits a particular propensity for tissues of the musculoskeletal system (8). In fact, *S. aureus* is among the most prominent causes of bacterial arthritis and is the single leading cause of osteomyelitis (8, 32). Additionally, *S. aureus* exhibits a remarkable capacity to colonize a wide variety of biomaterials, including those commonly used as orthopaedic implants (9).

The pathogenic diversity of *S. aureus* arises from its production of a diverse array of potential virulence factors, including extracellular proteins and proteins that remain exposed at the cell surface. With some exceptions (e.g., enterotoxin A) (30), these two groups are globally and inversely regulated, with the exoproteins being produced at the expense of specific surface proteins as the cells enter the postexponential phase of in vitro growth (13). The significance of this global shift to in vivo survival is unclear; however, mutants unable to undergo the shift to exoprotein synthesis have been shown to be less virulent in a number of animal models of staphylococcal disease (1, 3, 6, 27).

The regulatory events associated with the postexponential phase shift to exoprotein synthesis are complex and involve at least three regulatory loci. Specifically, the extracellular protein regulator (*xpr*) (27), the staphylococcal accessory regulator (*sar*) (4), and the accessory gene regulator (*agr*) (13) have all been shown to contribute to regulating expression of staphylococcal virulence factors. Mutations in *xpr* and *sar* have phenotypic effects similar to those observed in *agr* mutants (7, 27). Additionally, *xpr* and *sar* mutations have been shown to result in reduced expression of both RNAII and RNAIII (5, 10). Although the regulatory role of the *xpr* locus has not been examined for any *S. aureus* strain other than S6C (26, 27), the effect of mutations in *sar* appears to be at least somewhat strain dependent (4, 7). However, there is evidence, based on comparison of *sar* and *agr* mutations in the same genetic background, to suggest that *sar* and *agr* function through at least partially independent regulatory pathways (3). For instance, a recent study with two different *S. aureus* strains demonstrated that mutation of *sar* resulted in reduced expression of the fibronectin-binding proteins while mutation of *agr* had the opposite effect (3). Additionally, the *sar* mutation appeared to be dominant, in that strains carrying mutations in both regulatory genes exhibited a fibronectin-binding protein phenotype consistent with that of the same strains carrying mutations only in *sar* (3). Nevertheless, the observation that most *sar* mutants and the single *xpr* mutant so far examined exhibit phenotypes generally consistent with the phenotype of *agr* mutants suggests

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 W. Markham, Little Rock, AR 72205-7199. Phone: (501) 686-7958. Fax: (501) 686-5359. Electronic mail address: msmeltzer@biomed.uams.edu.

that the phenotypic effect of mutations in *sar* and *xpr* may be at least partly a consequence of their effects on expression of the genes encoded within *agr*. In any event, the fact that mutations in both *sar* and *xpr* result in reduced expression of the genes encoded within *agr* (5, 10) strongly suggests that the *agr* locus plays a central role in the regulatory events associated with the expression of many *S. aureus* virulence factors. This conclusion is supported by experimental evidence demonstrating the reduced virulence of *S. aureus agr* mutants in animal models of peritonitis, arthritis, and endocarditis (1, 3, 6, 27).

To further examine the role of the *agr* locus in the pathogenesis of musculoskeletal disease, we compared the virulence of an *S. aureus* isolate obtained from a patient with osteomyelitis caused by a derivative of the same strain carrying a mutation inactivating expression of *agr*. Virulence was assessed with a rabbit model of acute, exogenous osteomyelitis. We conclude from our results that mutation of the *agr* regulatory locus results in a significant reduction in both the incidence and severity of disease but does not eliminate the ability of *S. aureus* to colonize bone and cause localized evidence of osteomyelitis.

MATERIALS AND METHODS

Bacterial strains. The *S. aureus* strain designated UAMS-1 was isolated from a patient with osteomyelitis at the McClellan Veterans Hospital in Little Rock, Ark. The strain was deposited with the American Type Culture Collection and is available as strain ATCC 49230. UAMS-1 was maintained on tryptic soy medium without antibiotic selection. UAMS-4 was generated by φ11-mediated transduction from ISP546. ISP546 is a well-characterized strain that carries an *agrA*::Tn*551* insertion (18). Importantly, the Tn*551* insertion functionally inactivates the signal transduction system encoded within the RNAII transcript and consequently prevents induction of RNAIII expression (13, 15). Because Tn*551* encodes an erythromycin resistance determinant, UAMS-4 was maintained on tryptic soy medium containing 20 μg of erythromycin per ml. However, because repeated trials in which UAMS-4 was grown without selection and then examined for the presence of the *agrA*::Tn*551* insertion revealed no evidence of transposon instability (data not shown), UAMS-4 was grown without selection during the course of all phenotypic assays. *S. aureus* S6C, Cowan 1, and ISP479C have been described elsewhere (2, 11, 26, 27).

Genotypic and phenotypic characterization. Chromosomal DNA was isolated from *S. aureus* as described elsewhere (26). Total cellular RNA was isolated with RNAzol B as described by Hart et al. (10). Southern and Northern (RNA) blot analysis was done with digoxigenin-labelled DNA probes as previously described (10, 26, 27). Northern blot analysis was done with 10 μg of RNA per lane as determined from A_{260}/A_{280} ratios (10). DNA probes for Southern and Northern blot analysis were generated by PCR amplification with chromosomal DNA from *S. aureus* S6C (*sea* and *agr*), ISP479C (*hlb*), and UAMS-1 (the collagen adhesin, *cna*). The oligonucleotide primers used for each amplification have been described elsewhere (27–29).

Hemolytic activity was assessed by plating on tryptic soy agar containing 5% sheep or rabbit blood. The binding capacity for collagen and bone sialoprotein was determined with purified proteins labelled with ^{125}I. Type I collagen (from calf skin) was obtained commercially (Sigma Chemical Co., St. Louis, Mo.). Bone sialoprotein purified from a rat osteosarcoma cell line was a gift from Larry Fisher, National Institutes of Health. Proteins were labelled with ^{125}I by using Iodo-Beads (Pierce Chemical Co., Rockford, Ill). Aliquots (100 μCi; 10 μl) of ^{125}I (as sodium iodide in 0.1 N NaOH) were purchased from ICN Radiochemicals. After dilution of the iodine to 500 μl with 100 mM phosphate-buffered saline (PBS; pH 7.3), three Iodo-Beads were added and allowed to equilibrate for 5 min at room temperature. Then 500 μl of the appropriate protein diluted to 50 μg/ml in PBS was added, and the labelling reaction was allowed to proceed for 15 min at room temperature. The reaction was stopped by removing the labelling mixture from the Iodo-Beads. The 1.0-ml labelling mixture was applied to a PD-10 column (Pharmacia) preequilibrated with 25 ml of PBS. Labelled proteins were washed into the column with an additional 1.5 ml of PBS and then eluted with 3.5 ml of the same buffer. Fractions (0.5 ml) were collected, and the amount of radioactivity in each fraction was determined with a gamma counter. Labelled proteins consistently came off the column in fractions 2, 3, and 4 (data not shown). Specific activity ranged from 2.4×10^5 to 2.9×10^6 cpm/μg.

To determine the binding capacity for each host protein, each *S. aureus* strain was grown overnight in 20 ml of tryptic soy broth. Cells from the overnight cultures were harvested by centrifugation and washed with 20 ml of PBS containing 0.1% bovine serum albumin (BSA) and 0.1% Tween 80 (PBS-BSA-T). Cells were resuspended in 20 ml of PBS-BSA-T, and the A_{550} of each suspension was determined. Cell suspensions were standardized to the density of the least dense suspension by addition of an appropriate amount of PBS-BSA-T. Binding assays were done in 1.5-ml microcentrifuge tubes precoated with PBS containing 5% BSA (PBS-A). Tubes were precoated by being filled with PBS-A and incubated overnight at 4°C. For each ^{125}I binding assay, 3×10^5 cpm of labelled protein was added to 1.0 ml (approximately 3×10^9 CFU) of each cell suspension. Mixtures were continually rocked for 2 h at room temperature. Cells were then harvested by centrifugation, and the amount of radioactivity in the supernatant and in the cell pellet was determined with a gamma counter. For the collagen-binding assays, *S. aureus* S6C (a strain that does not encode *cna* [data not shown]) was used as a negative control. Cowan 1, a strain known to encode *cna* and exhibit a relatively high binding capacity for collagen (29), was used as a positive control. Because the gene encoding the *S. aureus* bone sialoprotein adhesin has not been identified, we were unable to identify a strain that could be clearly established as a negative control in our bone sialoprotein-binding assays. Results are therefore reported as the amount of protein bound relative to that observed with Cowan 1.

TABLE 1. Summary of trials with the rabbit model of acute, exogenous osteomyelitis

Trial	No. of rabbits/group	Infecting strain	Dose (CFU)	Inoculum vol (μl)
1	3	UAMS-1	1×10^7	50
	3	UAMS-4	1×10^7	50
	2	None	0	50
2	4	UAMS-1	2×10^6	10
	4	UAMS-4	2×10^6	10
	4	None	0	10
3	6	UAMS-1	2×10^6	10
	3	UAMS-1	2×10^5	10
	3	UAMS-1	2×10^4	10
	6	UAMS-4	2×10^6	10
	3	UAMS-4	2×10^5	10
	3	UAMS-4	2×10^4	10

Rabbit model for acute, exogenous osteomyelitis. For our osteomyelitis model, we used male, adult New Zealand White rabbits (2 to 3 kg each). *S. aureus* inoculum was prepared from overnight cultures grown in tryptic soy broth (UAMS-1) or tryptic soy broth containing 20 μg of erythromycin per cell (UAMS-4). Cells were harvested by centrifugation, washed with 20 ml of sterile physiological saline, and resuspended to a final density of 2×10^8 CFU/ml. Cell suspensions were held on ice during the course of the surgical procedures described below. Additionally, the density and purity of each cell preparation were verified by colony counts on selective and nonselective media both before and after each surgical session. Over the course of three independent trials, a total of 19 rabbits were infected with each strain while 6 served as sham-operated controls (Table 1). Of the 19 infected with each strain, 13 were infected with $\geq 2 \times 10^6$ CFU while 6 were infected with a lower dose ($\leq 2 \times 10^5$ CFU). The six controls were inoculated with sterile physiological saline.

Rabbits were fasted for 24 h prior to surgery. After sedation with 5 mg of xylazine per kg followed by 44 mg of ketamine per kg, the right forelimb was shaved and the rabbits were prepared for surgery with a Betadine scrub followed by a 70% ethanol rinse. The surgical site was painted with Prepodyne immediately before incision. During the surgical procedure, anesthesia was maintained with 3% halothane delivered by nose cone. An incision was made on the anterior surface of the right forelimb and extended through the epidermidis, musculature, and fascia until the radius was exposed. A jeweler's saw was then used to remove a 1-cm midradial segment for inoculation. In our first trial, a 50-μl inoculum containing 1×10^7 CFU was delivered by slowly dripping 25 μl onto each end of the excised bone segment. However, to obtain more precise control, the inoculum was reduced in subsequent trials to 10 μl (2×10^6 CFU) delivered by microinjection (with a sterile pipette tip with an outside diameter of 0.56 mm) directly into the center of the medullary canal. Importantly, reduction of the inoculum from 1×10^7 to 2×10^6 CFU had no effect on the infection rate obtained with UAMS-1 (data not shown). For delivery of lower doses ($\leq 2 \times 10^5$ CFU), the suspension standardized to 2×10^8 CFU/ml was diluted 1/10 (2×10^7 CFU/ml) or 1/100 (2×10^6 CFU/ml) to maintain the 10-μl inoculation volume. Sham-operated controls were given injections of 10 μl of sterile saline. After inoculation, the 1-cm midradial bone segment was replaced in its original orientation and the wound was closed. The halothane administration was discontinued, and the rabbits were observed until awake. Postoperative analgesic consisted of 0.05 mg of Buprenex per kg. Rabbits were monitored daily for 4 weeks, at which time they were sacrificed for bacteriological, radiographic, and histopathological analysis. Because the ulna was left intact, the rabbits maintained full mobility throughout the postinfection period. Additionally, although some

TABLE 2. Evidence of osteomyelitis in rabbits infected with UAMS-1 and UAMS-4

Data	Value for:				
	High dose ($\geq 2 \times 10^6$ CFU)		Low dose ($\leq 2 \times 10^5$ CFU)		Controls[a]
	UAMS-1	UAMS-4	UAMS-1	UAMS-4	
Bacteriological data[b]					
Reisolated	12 / 13	6 / 13	5 / 6	1 / 6	0 / 6
Intraosseous bacteria	12 / 13	6 / 13	6 / 6	1 / 6	0 / 6
Radiographic data[c]					
Periosteal elevation	2.69 ± 0.35	1.27 ± 0.20	1.50 ± 0.34	1.00 ± 0.26	1.17 ± 0.17
Architectural deformation	2.69 ± 0.40	0.64 ± 0.31	1.33 ± 0.42	0.33 ± 0.21	0.17 ± 0.17
Widening of bone shaft	2.69 ± 0.35	0.82 ± 0.30	1.33 ± 0.42	0.33 ± 0.21	0.17 ± 0.17
New bone formation	2.46 ± 0.21	1.18 ± 0.12	1.33 ± 0.33	1.00 ± 0.26	1.00 ± 0.00
Soft tissue deformation	2.69 ± 0.35	0.82 ± 0.33	1.67 ± 0.49	0.50 ± 0.22	0.17 ± 0.17
Composite score[d]	2.64 ± 0.30	0.95 ± 0.23	1.43 ± 0.39	0.63 ± 0.20	0.53 ± 0.08
Histopathological data[c]					
Intraosseous acute inflammation	3.69 ± 0.31	1.91 ± 0.61	3.33 ± 0.67	1.33 ± 0.71	0.17 ± 0.17
Intraosseous chronic inflammation	2.80 ± 0.23	1.91 ± 0.31	2.67 ± 0.33	1.33 ± 0.33	0.83 ± 0.17
Periosteal inflammation	1.92 ± 0.26	0.82 ± 0.26	0.67 ± 0.21	0.33 ± 0.21	0.17 ± 0.17
Bone necrosis	0.85 ± 0.30	0.36 ± 0.15	1.17 ± 0.48	0.33 ± 0.21	0.17 ± 0.17
Composite score[d]	2.31 ± 0.22	1.58 ± 0.29	1.96 ± 0.36	0.83 ± 0.32	0.33 ± 0.05
Composite scores (all animals)[c,e]					
Radiographic data	2.26 ± 0.27	0.84 ± 0.16			0.53 ± 0.08
Histopathological data	2.19 ± 0.19	1.31 ± 0.23			0.33 ± 0.05

[a] Control animals were sham operated and given a microinjection of 10 μl of sterile saline.
[b] Data given as number of rabbits with positive results/total number of rabbits.
[c] Results are reported as the mean score ± standard error of the mean.
[d] Composite score refers to the average score obtained for all radiographic or histopathological parameters.
[e] Composite scores observed with all animals (high plus low dose) in each infection group.

rabbits developed localized signs of infection (e.g., swelling over the site of incision), no rabbits exhibited signs of systemic disease during any part of the 4-week postinfection period.

At the end of the 4-week postinfection period, rabbits were sedated and then sacrificed by administration of 0.25 ml of Somlethol per kg delivered by intracardiac puncture. The entire right forelimb was surgically removed for analysis. After both anterior and lateral closed-system radiographs were obtained, the forelimb was surgically excised to expose the bone. Samples for bacteriological analysis were taken from the bone segment itself and from the adjacent soft tissue. Because the identity of the infecting strain was encoded, samples for bacteriological analysis were plated on tryptic soy agar with and without 20 μg of erythromycin per ml. The identity of individual isolates was confirmed by Southern blot analysis with DNA probes specific for *agr*, *hlb*, *sea*, and *cna*. After radiographs and bacteriological specimens were obtained, the remaining soft tissue was removed and the bones of the forelimb were placed in formalin for routine paraffin processing.

Radiographic and histopathological analysis. Investigators responsible for evaluating radiographs and stained histopathological sections were blinded with respect to the inoculum delivered to each rabbit. Additionally, to ensure continuity between trials, representative radiographs and slides from previous trials were included in each subsequent round of evaluation. Radiographic analysis was based on a five-point scale (0 to 4, with 4 representing the most severe), assessing evidence of (i) periosteal elevation, (ii) soft tissue deformation, (iii) architectural deformation, (iv) widening of the bone shaft, and (v) new bone formation. Scores for each parameter were averaged to derive a single composite score for each rabbit. For identification of intraosseous bacteria and histopathological analysis, paraffin blocks were cut at 6-μm intervals and stained with modified Gram stain (25) or with hematoxylin and eosin. Histopathological analysis was based on a five-point scale assessing evidence of (i) intraosseous acute inflammation, (ii) intraosseous chronic inflammation, (iii) periosteal inflammation, and (iv) bone necrosis. Scores for each parameter were averaged to obtain a single composite score for each rabbit. Results of the radiographic and histopathological analyses are reported as the mean ± standard error of the mean (Table 2). Statistical analysis of the results was done by Student's paired *t* test. $P \leq 0.05$ was considered statistically significant (Table 3).

RESULTS

Characterization of UAMS-1 and UAMS-4. Chromosomal DNA from UAMS-1, six erythromycin-resistant (Erm^r) transductants (lanes 1 to 6), ISP479C (wild-type parent of ISP546), and ISP546 (*agrA*::Tn*551*) was digested with *Eco*RV and probed with DNA fragments that collectively span the *agr* locus (Fig. 1A). The 4.1-kbp fragment present in UAMS-1 and ISP479C was previously shown to include the region encoding *agrA* (18, 27). As evidenced by comparison with ISP546, all six UAMS-1 transductants carried the 5.3-kbp transposon in the 4.1-kbp fragment encoding *agrA*. Additionally, UAMS-1 exhibited an *Eco*RV polymorphism resulting in replacement of the 1.2-kbp fragment present in ISP479C and ISP546 with a 3.0-kbp fragment (Fig. 1A). This polymorphism, which is due to the absence of an *Eco*RV site in the region downstream from the region of the *agr* locus encoding RNAIII (18, 27), was not present in five of the six transductants. However, like UAMS-1, all six transductants were subsequently shown to be lysogenized with an *hlb*-converting bacteriophage that encodes *sea*

TABLE 3. *P* values for radiographic and histopathological parameters

Data	*P* value for:		
	UAMS-1 vs controls	UAMS-4 vs controls	UAMS-1 vs UAMS-4
Radiographic data			
High dose	0.018	0.150	0.003
Low dose	0.088	0.718	0.047
All animals	0.018	0.150	0.002
Histopathological data			
High dose	0.013	0.025	0.030
Low dose	0.010	0.216	0.040
All animals	0.012	0.025	0.004

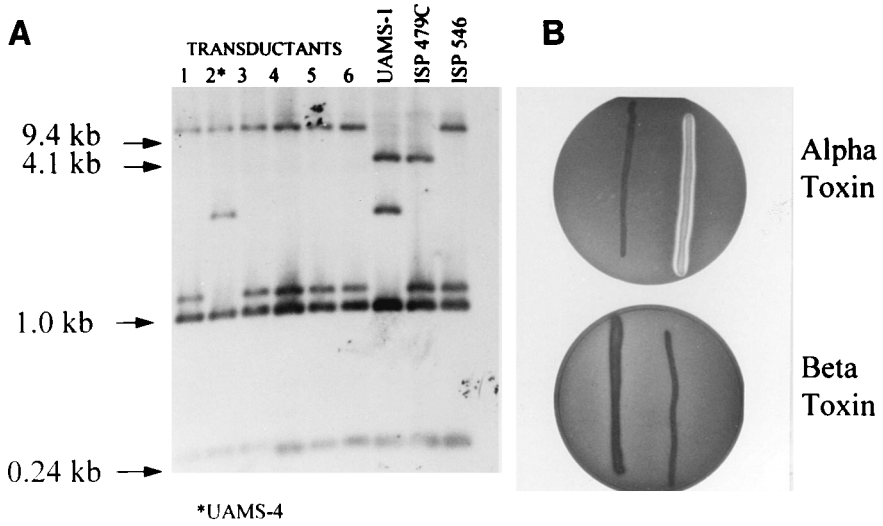

FIG. 1. Characterization of *S. aureus* UAMS-4. (A) Chromosomal DNA from UAMS-1, ISP479C (the parent strain of ISP546), ISP546 (a mutant of ISP479C carrying a Tn*551* insertion within the *agr* locus), and six strains obtained by ϕ11-mediated transduction with ISP546 as the donor and UAMS-1 as the recipient (lanes 1 to 6) was digested with *Eco*RV and probed with a 1.75-kbp DNA known to hybridize to all four *Eco*RV fragments encoded within *agr*. The 1.2-kbp fragment present in most strains is defined by an *Eco*RV site within the region encoding RNAIII and a second site located outside the *agr* locus (26). The fact that the 1.2-kbp fragment is replaced by a larger fragment in UAMS-1 is indicative of a polymorphism arising from an *Eco*RV site outside the *agr* locus. Although all six transductants were subsequently shown to be derivatives of UAMS-1 (Fig. 2) and to exhibit an Agr^- phenotype (data not shown), the transductant designated UAMS-4 (lane 2) was chosen for further study because the *Eco*RV polymorphism confirming its UAMS-1 heritage was retained. (B) Hemolytic activity of UAMS-1 (right) and UAMS-4 (left) on rabbit (top) and sheep (bottom) blood agar. The lack of α-toxin production in UAMS-4 (top) is characteristic of strains carrying mutations inactivating the *agr* locus. Neither strain is hemolytic on sheep blood, because they are lysogenized with a β-toxin-converting bacteriophage (data not shown).

and to encode a *cna* gene with a single B domain (references 16, 17, and 29 and data not shown). Although these results strongly suggest that all six transductants are derivatives of UAMS-1, the single transductant that retained the *Eco*RV polymorphism was chosen for further analysis because the presence of the polymorphism provided a direct indication of the UAMS-1 genetic background. This transductant was subsequently designated UAMS-4. Inactivation of the *agr* locus was confirmed by demonstrating that UAMS-4 failed to produce detectable amounts of α-toxin (Fig. 1B) or either of the two transcripts (RNAII and RNAIII) encoded within *agr* (Fig. 2).

The *agr*::Tn*551* insertion in UAMS-4 did not affect its in vitro growth rate in either the presence or absence of antibiotic selection (Fig. 3). Additionally, the high binding capacity of UAMS-1 for collagen was not significantly affected by mutation of *agr* (Table 4). Neither UAMS-1 or UAMS-4 bound appreciable amounts of bone sialoprotein (Table 4).

Virulence of UAMS-1 and UAMS-4 in the osteomyelitis model. Pure cultures of *S. aureus* were isolated from 17 of 19 rabbits infected with UAMS-1 and 7 of 19 rabbits infected with UAMS-4 (Table 2). These results were consistent with our Gram stain analysis confirming the presence of gram-positive, intraosseous cocci in 18 of 19 rabbits infected with UAMS-1 and 7 of 19 infected with UAMS-4 (Table 2). The isolation rate with respect to UAMS-4 was clearly dose dependent, with 6 of 7 isolates being obtained from rabbits infected with $\geq 2 \times 10^5$ CFU (Table 2). No bacteria were isolated from any of the control rabbits. With one exception, all isolates from rabbits infected with UAMS-1 failed to grow on medium containing erythromycin. In one case, a small number ($\leq$25) of Erm^r colonies were isolated from a rabbit infected with UAMS-1. Southern blot analysis with gene probes for *agr* (Fig. 4), β-toxin

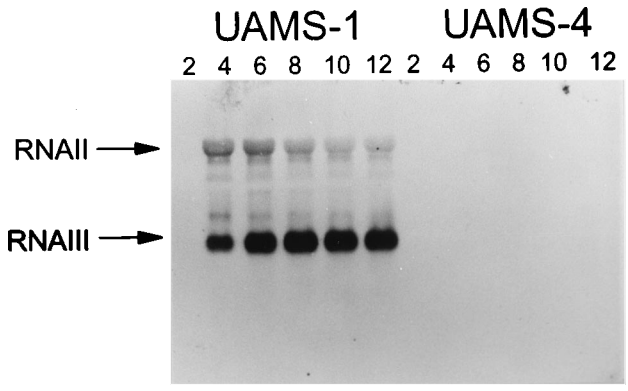

FIG. 2. Northern blot analysis of RNAII and RNAIII expression. Total cellular RNA was isolated from cultures of UAMS-1 and UAMS-4 at the time points indicated (lane assignments represent hours of in vitro culture). Growth was at 37°C with constant rotation in tryptic soy broth. UAMS-4 was grown without selection. RNA was electrophoresed and probed with DNA fragments that collectively span the *agr* locus.

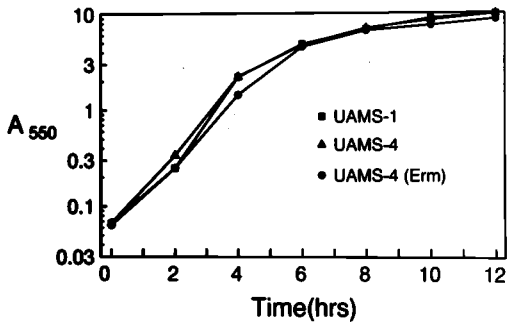

FIG. 3. Growth of UAMS-1 and UAMS-4 in vitro. UAMS-1 was grown in tryptic soy broth, while UAMS-4 was grown in TSB with and without 20 μg of erythromycin per ml.

TABLE 4. Binding capacity for collagen and bone sialoprotein

Strain	Relative binding capacity of strain for ^{125}I-labelled protein:	
	Collagen	Bone sialoprotein
Cowan 1[a]	1.00	1.00
UAMS-1	1.23	1.33
UAMS-4	1.20	1.06
S6C[b]	0.18	27.24

[a] Values for Cowan 1 were set at 1.00. Values for other strains reflect the binding capacity relative to that observed in Cowan 1.

[b] S6C was used as a negative control in the collagen-binding assays because it does not encode *cna* (data not shown).

(*hlb*), enterotoxin A (*sea*), and the collagen adhesin (*cna*) confirmed that these Erm^r colonies were contaminants unrelated to UAMS-1 (data not shown). Although our bacteriological analysis was not quantitative, it should be noted that the number of Erm^r colonies was very small compared with the number of Erm^s colonies (data not shown). Given the overwhelming predominance of Erm^s colonies and the probability that the Erm^r contaminant was acquired during the evaluation surgery, the rabbit from which this contaminant was obtained was not excluded from the subsequent radiographic and histopathological analysis. All isolates from rabbits infected with UAMS-4 were Erm^r. However, Southern blot analysis confirmed that two of these isolates encoded intact *agr* loci, did not exhibit the *Eco*RV polymorphism characteristic of the *agr* locus encoded by UAMS-1, did not carry a β-toxin-converting bacteriophage, and did not encode any form of *cna* (data not shown). For that reason, these rabbits were excluded from further radiographic and histopathological analysis (i.e., they were not used to produce the data presented in Table 2). However, because our Southern blot analysis of the Erm^r isolates from these two rabbits revealed no evidence of UAMS-4 (i.e., the Southern blot patterns obtained with these isolates were unambiguous), we assumed the absence of UAMS-4 and included these rabbits when determining the overall isolation rate for each strain (Table 2). That assumption was supported by our failure to observe intraosseous bacteria in gram-stained bone sections from either rabbit.

Our bacteriological results were consistent with the results of our radiographic analysis (Fig. 5). Specifically, each of five parameters (periosteal elevation, soft tissue deformation, architectural deformation, new bone formation, and widening of the bone shaft) was scored on a scale of 0 to 4. The five scores obtained for each rabbit were then averaged to obtain a single, composite radiographic score. Rabbits infected with UAMS-1 had an average composite radiographic score of 2.26 ± 0.27 with a range of 2.64 ± 0.30 (high dose) to 1.43 ± 0.39 (low dose) (Table 2). In contrast, rabbits infected with UAMS-4 had an average composite radiographic score of 0.84 ± 0.16 with a range of 0.95 ± 0.23 (high dose) to 0.63 ± 0.20 (low dose). Control rabbits had an average radiographic score of 0.530 ± 0.08 (Table 2). The average composite scores obtained with rabbits infected with either dose of UAMS-1 were significantly different from the scores obtained with rabbits infected with UAMS-4 and from the control animals (Table 3). The difference observed between rabbits infected with UAMS-4 and the controls was not statistically significant regardless of the UAMS-4 dose used (Table 3).

The difference between rabbits infected with UAMS-1 and those infected with UAMS-4 was confirmed in our histopathological analysis (Fig. 6; Table 2). Specifically, rabbits infected with UAMS-1 had an average composite score of 2.19 ± 0.19

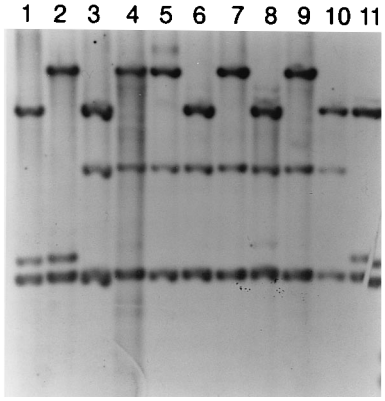

FIG. 4. Chromosomal DNA from *S. aureus* ISP479C (lane 1), ISP546 (lane 2), UAMS-1 (lane 3), UAMS-4 (lane 4), and six isolates obtained from rabbits infected with UAMS-1 (lanes 6, 8, and 10) or UAMS-4 (lanes 5, 7, and 9) was digested with *Eco*RV and probed with an *agr*-specific DNA fragment as described in the legend to Fig. 1. Lane 11 contains chromosomal DNA from the Erm^r contaminant described in the text.

with a range between 2.31 ± 0.22 (high dose) and 1.96 ± 0.36 (low dose) while rabbits infected with UAMS-4 had an average score of 1.31 ± 0.23 with a range between 1.58 ± 0.29 (high dose) and 0.83 ± 0.32 (low dose). Controls had an average composite score of 0.33 ± 0.05. With regard to the overall scores and the scores obtained with rabbits inoculated with the higher dose, the differences between all groups were statistically significant (Table 3). The scores obtained with rabbits infected with the lower dose of UAMS-4 were not statistically different from those for the controls (Table 3).

DISCUSSION

S. aureus encodes a diverse array of virulence factors, many of which are coordinately regulated (19, 27). This coordinate regulation is a complex process that appears to involve at least three distinct loci (7, 19, 27). The best characterized of these is the accessory gene regulator (*agr*). The *agr* locus encodes two divergent transcripts, one of which (RNAII) is thought to encode a two-component signal transduction system (13). Although certain environmental signals have been shown to affect expression of the *agr* locus (14, 20–23, 31), the precise nature of the signal transduction system encoded within *agr* remains unclear. However, the fact that mutations in the region of *agr* encoding the putative signal transduction system essentially eliminate RNAIII expression clearly indicates that the promoter for the RNAIII transcript is responsive to signals transmitted either directly or indirectly through the signal transduction system encoded within RNAII (13, 19, 27). Additionally, while the RNAIII transcript includes the structural gene encoding staphylococcal δ-toxin (12), a number of studies have demonstrated that it is the RNAIII transcript itself that exerts the regulatory effect associated with expression of the *agr* locus (13, 15). In fact, RNAIII has been shown to be necessary, although not always sufficient, for the shift to exoprotein synthesis (15, 31). Importantly, the shift to exotoxin synthesis is associated with a concomitant decrease in the expression of a variety of proteins normally associated with the staphylococcal cell surface. Most notable are coagulase and protein A, both of which are overexpressed in *agr* mutants (13, 27). Additionally, it was recently demonstrated that the binding capacities for vitronectin and fibronectin are enhanced by mutation of *agr* (1). In contrast, the binding capacity of *S.*

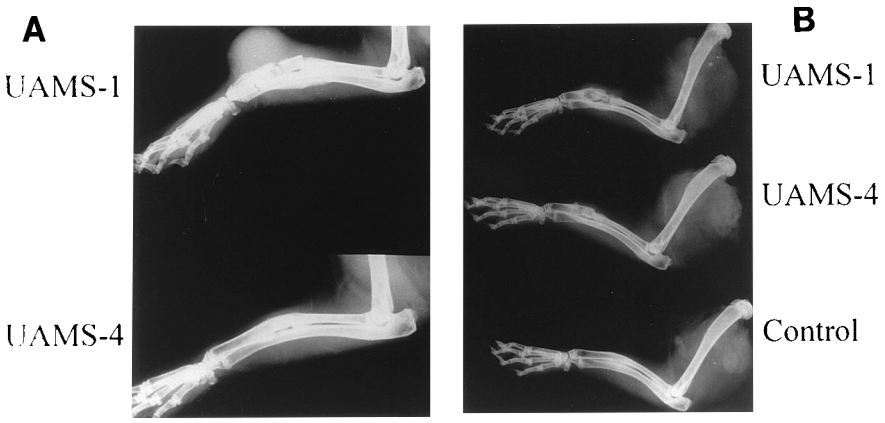

FIG. 5. Radiographic evidence of osteomyelitis. Rabbits were infected as described in Materials and Methods with 10^7 CFU (A) or 2×10^6 CFU (B) of UAMS-1 or UAMS-4. Radiographs were obtained after 4 weeks.

aureus 8325-4 for bone sialoprotein was reduced in *agrA* mutants (1). To date, the effect of *agr* mutation on the binding capacity for collagen in a strain that exhibits a high binding capacity for collagen has not been examined. However, our results clearly demonstrate that mutation of the *agr* locus in UAMS-1, a strain that exhibits a binding capacity greater than that observed with the prototypical strain Cowan 1 (16, 17), had little or no effect on the binding capacity for type I collagen.

Although the in vivo significance of the temporal regulation of surface proteins and the global shift to toxin synthesis is unclear, it is tempting to speculate that staphylococcal surface proteins are expressed early in the course of infection during the process of colonization whereas toxins are produced in response to changing physiological conditions at a localized site of infection (13, 15). Such a scenario implies that mutation of the *agr* locus, which characteristically results in an exoprotein-deficient phenotype, would inhibit the ability of *S. aureus* to persist at a localized site of infection. Our results are consistent with that hypothesis. Specifically, we confirmed that UAMS-1 was present in the bone of 18 of 19 infected rabbits (95%) 4 weeks after direct introduction into the medullary cavity while UAMS-4 was present in only 7 of 19 (37%). These results are even more significant when considered on a dose-dependent basis. For example, while bacteria were observed in the bone of all rabbits infected with $\leq 2 \times 10^5$ CFU of UAMS-1, their presence was confirmed in the bone of only one of six rabbits infected with the same dose of UAMS-4. These results clearly suggest that mutation of the *agr* locus significantly reduces the ability of *S. aureus* to persist in the bone. There are two possible explanations for this reduced ability to persist in vivo. First, assuming a role for exotoxins in maintaining infection at localized sites, *agr* mutants would be less persistent by virtue of their inability to shift to exotoxin synthesis in response to changing physiological conditions in the host. Although the precise role of exotoxins in the pathogenesis of osteomyelitis has not been investigated, it is possible that exotoxins contribute to survival by virtue of their ability to promote tissue degradation and thereby enhance nutrient acquisition and/or the ability to ward off host defenses. Alternatively, the observation that *agr* mutants express many surface proteins at levels that meet or exceed wild-type levels suggests that such mutants may be more susceptible to phagocytosis. Interestingly, in a preliminary study in which we infected three rabbits with equal numbers (10^6 CFU) of both UAMS-1 and UAMS-4, we were unable to isolate UAMS-4 from any rabbit although all rabbits were colonized with UAMS-1 and exhibited signs of osteomyelitis (data not shown). On the basis of the observation that sufficient exotoxin was produced to maintain the UAMS-1 infection and the presumption that the presence of these exotoxins in the immediate environment would have the same effect on all staphylococci in that environment, these results support the hypothesis that *agr* mutants are inherently more susceptible to host clearance systems. However, it remains noteworthy that we isolated UAMS-4 from 46% of rabbits infected with $\geq 2 \times 10^6$ CFU and 17% of those infected with $\leq 2 \times 10^5$ CFU. While these results are difficult to interpret in the absence of a clear understanding of the role played by surface proteins in the pathogenesis of osteomyelitis, the fact that bacteria were present in the bone after 4 weeks of in vivo incubation strongly suggests that mutation of *agr* did not eliminate the ability of *S. aureus* to persist in the bone and cause localized signs of clinical disease. Our demonstration that expression of the UAMS-1 collagen adhesin was not affected by mutation of *agr* may be relevant in that regard.

The results presented here demonstrate an important role for the *agr* locus in staphylococcal osteomyelitis and, together with previous reports demonstrating the reduced virulence of *agr* mutants in a murine model for septic arthritis (1), strongly suggest that the *S. aureus agr* locus is an important virulence determinant in the pathogenesis of musculoskeletal disease. This observation clearly implies that therapeutic strategies aimed at inhibiting expression of *agr* could reduce both the incidence and severity of musculoskeletal disease. However, it should be emphasized that mutation of the *agr* locus did not eliminate the ability to cause disease in either our model or the murine arthritis model (1). That observation implies that virulence factors that either are not regulated by *agr* or are negatively regulated (such that they are overexpressed in an *agr* mutant) also contribute to disease. In the case of UAMS-4, that residual pathogenicity could arise from the fact that enterotoxin A, which is encoded within the UAMS-4 genome by virtue of a β-toxin-converting bacteriophage, is not regulated by *agr* (30). Alternatively, the expression of specific staphylococcal surface proteins (e.g., the collagen adhesin) at or above wild-type levels may enhance the ability to colonize specific tissues to the point that the organism can persist in the host in the absence of overt disease. This is a particularly relevant consideration in light of the fact that staphylococcal musculoskeletal infections are often chronic and become clinically obvious only after prolonged periods.

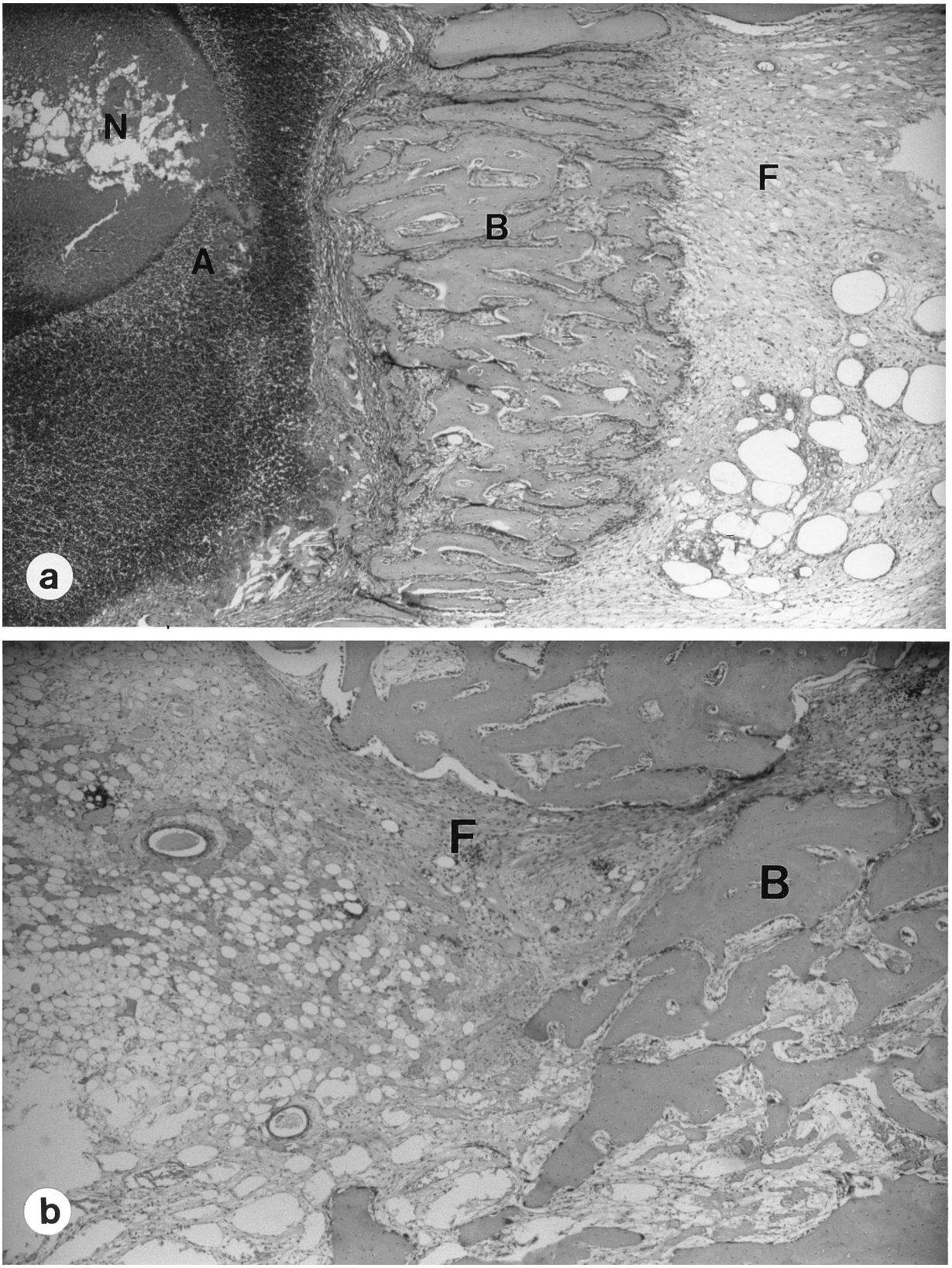

FIG. 6. Histopathological evidence of osteomyelitis. Stained sections from a severely affected rabbit (a) and a rabbit showing limited evidence of disease (b) are shown for comparison. Specific signs of osteomyelitis include an abscess (A) containing necrotic tissue (N), new bone formation (B) indicative of an attempt to wall off the abscess, and fibrosis (F) indicative of a chronic inflammatory process. The new bone formation shown in panel b is probably due to normal processes of postsurgical repair.

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